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Note

Simultaneous determination of glucosamine and glucosamine 4-phosphate in Lipid A with high-performance anion-exchange chromatography (HPAEC)[1]

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Abstract

A method for simultaneous determination of glucosamine (GlcN) and glucosamine 4-phosphate (GlcN-4-P) in Lipid A with high-performance anion-exchange chromatography (HPAEC) is described. Lipid A is hydrolyzed with 4 M HCl for 16 h at 100 °C, and the peaks of glucosamine and glucosamine 4-phosphate were measured. The true GlcN value can be computed from the GlcN value after correction for the incomplete hydrolysis of GlcN-4-P, or by the combined yield of GlcN and GlcN-4-P.

Keywords: Glucosamine; Glucosamine-4-phosphate; HPAEC; Lipid A; Analysis

The Lipid A in all lipopolysaccharides (LPS [1]) of Gram-negative bacteria contains β-GlcN-(1→6)-GlcN, which is both *N*- and *O*-acylated [2,3]. Moreover, the GlcN residue at the reducing terminus contains a glycosidic phosphate (1-phosphate), and GlcN at the non-reducing terminus contains 4′-phosphate. Because of the importance of Lipid A in many medical problems, accurate analytical methods for Lipid A is in high demand.

We have developed an effective method of determination of Kdo [4], using mildly acidic conditions for selective hydrolysis of the ketosidic linkage of Kdo. Although the glycosidic phosphate is extremely acid-sensitive, the other phosphate (4′-phosphate) is more resistant to hydrolysis. The usual conditions for complete release of GlcN

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Abbreviations: LPS, lipopolysaccharides; Kdo,3-deoxy-D-*manno*-2-octulosonic acid; HPAEC, high performance anion exchange chromatography; GlcNAc-1-P. *N*-acetyl-α-D-glucosamine-1-phosphate.;GlcNAc-6-P; *N*-acetyl-glucosamine 6-phosphate; Man-6-P, mannose 6-phosphate; MeGlcNAc-4-P, methyl 2-acetamido-2-deoxy-4-*o*-phosphoryl-β-D-glucopyranoside.

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from glycoproteins [5] (6 h in 4 M HCl at 100 °C) *N*-deacylate both GlcN residues in Lipid A, but less than 40% of the total GlcN was released from Lipid A, with GlcN 4-phosphate being the major intermediate. Therefore, for accurate determination of GlcN in Lipid A, both GlcN and GlcN 4-phosphate (GlcN-4-P) must be measured. In the method described here, we use HPAEC to determine GlcN and GlcN-4-P simultaneously in less than 25 min. A reference compound, methyl 2-acetamido-2-deoxy-4-*O*-phosphoryl-β-D-glucopyranoside (Me-GlcNAc-4-P), was chemically synthesized for this purpose.

1. Materials

D-Glucosamine hydrochloride and *N*-acetyl-D-glucosamine were from Pfanstiehl Laboratories, Inc. (Waukegan, IL). *N*-acetyl-D-glucosamine 6-phosphate (GlcNAc-6-P), *N*-acetyl-α-D-glucosamine 1-phosphate (GlcNAc-1-P), and D-mannose 6-phosphate (Man-6-P) were from Sigma Chem.Co. (St. Louis, MO). Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Me-GlcNAc) was prepared in our laboratory. Preparation of LPS from *Salmonella minnesota* R595 and its *O*-deacylated form (DeOA-LPS) have been described [4].

2. Methods

Phosphate analysis.—Organic phosphate was measured by the method of Ames and Dubin [6], using Man-6-P as standard. Inorganic phosphate generated from glycosidic phosphate after hydrolysis by mild acid was determined by the ammonium molybdate method [7].

High performance anion-exchange chromatography.—An HPAEC system (Dionex BioLC) equipped with a pulsed amperometric detector (PAD-II), were used with an anion-exchange column (Carbopac PA-1, 4.6×250 mm) [4]. The effluent was mixed with 0.3 M NaOH at the rate of 1 mL/min to maintain the basicity required for the sensitivity of the detector. Areas under the curves were integrated with a Spectra-Physics (SP4270) or AI-450 (Dionex) integrator. For simultaneous analysis of GlcN and GlcN-4-P, the gradient scheme shown in Table 1 was used.

Synthesis of methyl 2-acetamido-2-deoxy-4-O-phosphoryl-β-D-glucopyranoside (Me-GlcNAc-4-P).—Methyl 2-acetamido-3,6-di-*O*-benzoyl-2-deoxy-β-D-glucopyranoside [8] (133 mg, 0.3 mmol) in tetrahydrofuran (3 mL) was treated with $POCl_3$ (70 μL, 0.75 mmol) and Et_3N (139 μL, 1 mmol) at 0 °C for 10 h. Ice–water (5 mL) was added and the mixture stirred for 12 h at room temperature. After adjusting the pH to 2, the mixture was extracted with $CHCl_3$ (4×8 mL), and the combined extracts were evaporated. The residue was treated with 2 M NaOH (3 mL) for 2 h, at room temperature for 2 h, and the solution was applied to a column of Dowex 50W-X8 (H^+-form, 1.5×10 cm) and the column was eluted with water. Fractions containing carbohydrates were combined and extracted with ether (2×3 mL) to remove benzoic acid. The aqueous solution was concentrated and applied to a column of Sephadex G-10 (1.5×90 cm) which was pre-equilibrated and washed with 50 mM HOAc. Fractions (2 mL) containing the desired product (revealed by TLC) were pooled and lyophilized to give the 4-phosphate (32 mg, 34%) as a white foam: R_F 0.22 (3:2:1 EtOAc–HOAc–water, E. Merck silica gel plate); m.p. 150 °C (dec.); LSI-MS (negative mode): 314[M-H]$^-$; ^{1}H-NMR (300 MHz, D_2O): 4.414 (1 H, twisted d, *J* 8.2 Hz, H-1), 3.875 (1 H, q, *J* 9.3 Hz, H-4), 3.872 (1 H, dd, *J* 2.1 and 12.5 Hz, H-6a), 3.740 (dd, 1 H, *J* = 5.4 and 12.5 Hz, H-6b), 3.710–3.681 (m, 2 H, H-2,3), 3.550–3.480 (m, 1 H, H-5), 3.458 (s, 3 H, OMe), 1.982 (s, 3 H, NAc).

Table 1
HPAEC conditions for GlcN and GlcN phosphate

Time	NaOH (0.20 M) (%)	Water (%)	NaOAc (1 M) (%)
0	40	60	0
10	40	60	0
22	40	10	50
24	40	10	50
30	40	60	0
40	40	60	0

3. Results

Mild acid hydrolysis of GlcNAc-1-P, GlcNAc-6-P, and Me-GlcNAc-4-P.—As expected, GlcNAc was released readily from GlcNAc-1-phosphate with 0.1 M HCl within 2 h at 100 °C (Fig. 2A). Less GlcNAc was released from Me-GlcNAc-4-P under the same conditions, and even less was liberated from GlcNAc-6-P (Fig. 2B).

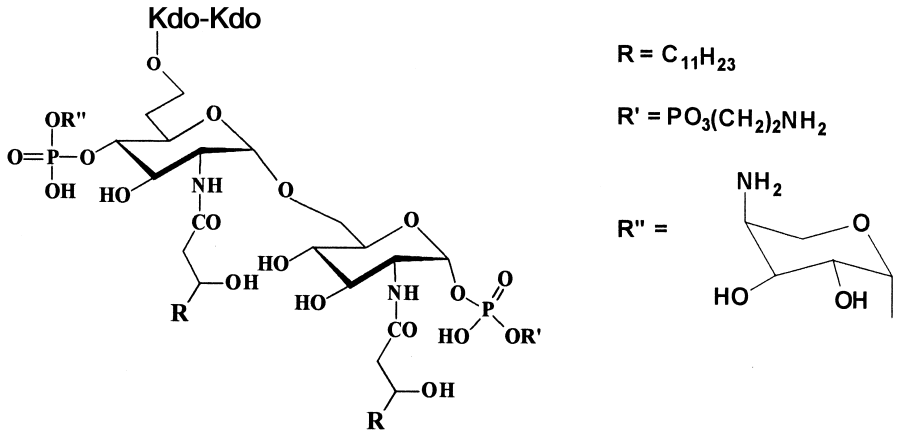

Fig. 1. Diagram of DeOA-LPS [4] generated from *Salmonella minnesota* R595 LPS by mild hydrazinolysis. The R is usually an alkyl chain of $C_{9\text{-}11}$, and R' group can be H or *O*-phosphorylethanolamine. [3,9]

Extended hydrolysis of Me-GlcNAc-4-P, and DeOA-LPS.—The time-courses of extended hydrolysis of Me-GlcNAc and Me-GlcNAc-4-P (Fig. 3) with 4 M HCl at 100 °C were studied. Under the same condition of hydrolysis, Me-GlcNAc released the maximum amount of GlcN after 2 h, and the released GlcN was stable up to 16 h. Although the peak of Me-GlcNAc-4-P disappeared after 2 h of heating under these conditions, the hydrolyzate after 6 h of heating showed two intermediate peaks, *X* and *Y* (Fig. 4), which were also seen in the hydrolyzate of DeOA-LPS at 6 h (Fig. 4, inset). These peaks disappeared almost totally after 16 h of hydrolysis and are presumably intermediates leading to GlcN-4-P (Fig. 3).

4. Discussion

Accurate determination of GlcN in Lipid A is complicated by the resistance of non-glycosidic phosphates to acid hydrolytic conditions. Using 4 M HCl for 6 h at 100 °C, the usual hydrolytic conditions for complete release GlcN from glycoproteins and glycolipids [5], generation of GlcN from GlcNAc-6-P and Me-GlcNAc-4-P was slow and incomplete (Fig. 2B). Moreover, after 6 h of hydrolysis of Me-GlcNAc-4-P, there were two peaks (*X* and *Y*) near the peak of GlcN-4-P. These peaks decreased in size upon further hydrolysis, and after 16 h, they became negligible.

Using the chromatographic conditions described here, GlcN-4-P is separable from GlcN (Fig. 4), and with the availability of a reference compound, Me-GlcNAc-4-P, calibration of recovery from GlcNAc-4-phosphate is possible. Thus the composition of the GlcN in the Lipid A can be computed by one of the following two methods.

(i) By use of a recovery factor: Since liberation of GlcN from the GlcN-1-P in the Lipid A backbone of 4′-phospho-β-GlcN-(1→4)-GlcN is accomplished at 100 °C with 4 M HCl, and GlcN is stable

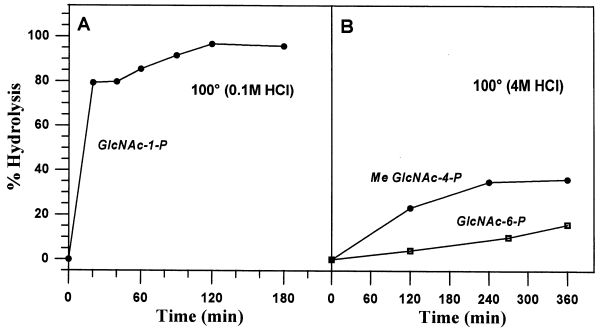

Fig. 2. Hydrolysis of GlcNAc-1-P and GlcNAc-6-P. (A) Hydrolysis (100 °C in 0.1 M HCl) of GlcNAc-1-P measured by the released GlcNAc (-●-); (B) Hydrolysis (4 M HCl at 100 °C) of Me-GlcNAc-4-P (-●-) and GlcNAc-6-P (-□-), measured by the release of GlcN.

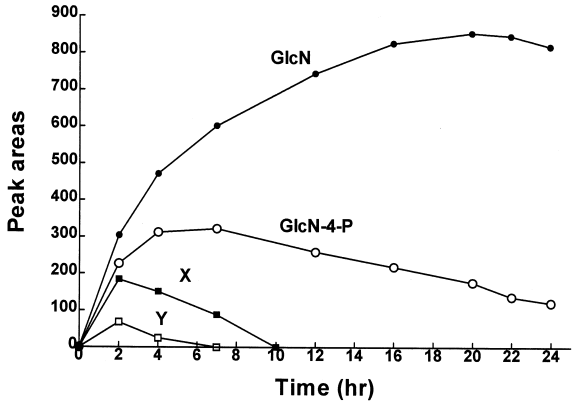

Fig. 3. Time course of extended hydrolysis of Me-GlcNAc-4-P. GlcN, (-●-); GlcN-4-P, (-▲-); Peak X (-■-); Peak Y (-□-).

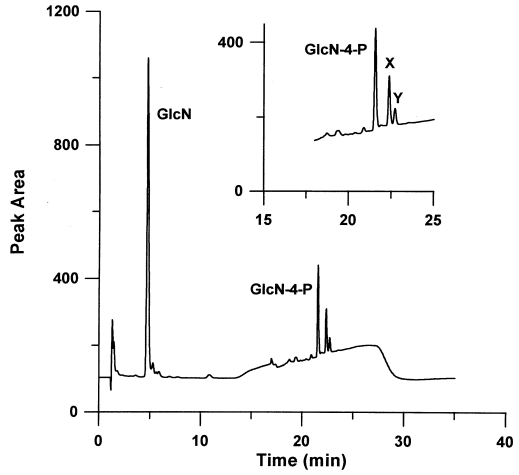

Fig. 4. HPAEC Profiles of hydrolyzates of Me-GlcNAc-4-P (Fig. 4) and DeOA-LPS (Fig. 4, inset) at 100 °C with 4 M HCl for 6 h.

throughout the hydrolysis up to 16 h, the GlcN content in Lipid A can be calculated as follows:

$$\text{GlcN value} = 2 \times (\text{observed GlcN})/(1 + \text{recovery factor})$$

where "recovery factor" is the yield of GlcN from Me-GlcNAc-4-P, and is experimentally determined (under the conditions described here, 87.5% after 16 h of hydrolysis). The results from 16 h of hydrolysis with 4 M HCl at 100 °C are preferable if such a computation is to be used, because the intermediate peaks became negligible after 16 h.

(ii) From the combined yield of GlcN and GlcN-4-phosphate; the molar response of GlcN-phosphate in the PAD-II system was estimated to be the same as that of GlcN, because GlcNAc and GlcNAc 6-phosphate was found be nearly equal in molar detector response. Therefore, the sum of GlcN and GlcN-4-P can be assumed to be equal to the total amount of GlcN present in Lipid A.

References

[1] U. Zähringer, B. Rindner, and E.T. Rietschel, *Adv. Carbohydr. Chem. Biochem.*, 50 (1994) 211–276.
[2] H. Brade, L. Brade, and E.T. Rietschel, *Zbl. Bakt. Mikrobiol. Ser. A.*, 268 (1988) 151–179.
[3] C.R. Raetz, *Annu. Rev. Biochem.*, 59 (1990) 129–170.
[4] J. Kiang, S. Szu, L.X. Wang, M. Tang, and Y.C. Lee, *Anal. Biochem.*, 245 (1997) 97–101.
[5] J.-Q. Fan, Y. Namiki, K. Matsuoka, and Y.C. Lee, *Anal. Biochem.*, 219 (1994) 375–378.
[6] B.N. Ames and D.T. Dubin, *J. Biol. Chem.*, 235 (1960) 758–769.
[7] P.S. Chen, T.Y. Toribara, and W. Warner, *Anal. Chem.*, 28 (1956) 1756–1758.
[8] L.X. Wang and Y.C. Lee, *J. Chem. Soc., Perkin Trans. I.*, (1996) 581–591.
[9] R.T. Rietschel et al., *FASEB J.* 8 (1994) 217–225.